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(57) Abstract

An anti-inflammatory preparation comprises a purified, active fraction isolated from a lipid extract of Perna canaliculus or Mytilus edulis, or an active component thereof. The compound 5,11,14,17-eicosatetraenoic acid may be a major constituent of the active fraction. A substantially pure form of 5,11,14,17-eicosatetraenoic acid is isolated and pharmaceutically acceptable esters, amides and salts thereof identified. These compounds are also used in anti-inflammatory preparations and treatments.

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ANTI-INFLAMMATORY PREPARATION

This invention relates in general to an anti-inflammatory preparation, and in particular it relates to an anti-inflammatory preparation isolated and identified from lipid extracts of mussels, including the New Zealand green lipped mussel, *Perna canaliculus*, and the blue mussel *Mytilus edulis*.

There is at the present time a significant medical need for new anti-inflammatory drugs with reduced side effects and prolonged *in vivo* activity and in particular for compounds which will moderate the progress of the arthropathies. Plants and other living cells offer a vast reservoir of compounds which have pharmacological effects on humans. Natural products have frequently been the source of effective drugs and lately there has been an increased interest in the analysis of these natural products, especially where a clinical benefit is claimed. Marine organisms contain metabolites that can act as pharmacological agents and aid in the treatment of inflammation.

An anti-inflammatory activity of *Perna canaliculus* (New Zealand Green Lipped Mussel) was first implicated as part of a pharmacological study on leukaemia. Initial assessment of the anti-inflammatory activity of *Perna canaliculus* was first attempted using a polyarthritis model in rats[1]. These studies however failed to show the presence of any significant anti-inflammatory activity in the mussel preparation. In contrast, Miller and Ormrod[2] using a carrageenan-induced paw oedema assay[3], were able to show that mussel preparations, when administered intraperitoneally, gave a significant reduction in the swelling of a carrageenan-induced rat paw oedema. Subsequently, they fractionated a non-dialysable, water-soluble fraction from the mussel preparation that possessed anti-inflammatory activity. The aqueous extract showed a dose-dependent anti-inflammatory activity when administered intraperitoneally and could not be detected upon oral administration of the mussel powder. It was suggested that

the water-soluble fraction therefore contained an irritant component possessing apparent anti-inflammatory activity.

Rainsford and Whitehouse[4] also reported that freeze-dried powdered preparations of the whole mussel given orally to rats showed some modest anti-inflammatory activity in the carrageenan-induced paw oedema assay, and that this material strikingly reduced the gastric ulcerogenicity of several non-steroidal anti-inflammatory drugs in rats and pigs.

Later investigations by the present inventors using isolated acid and neutral lipid extracts from *Perna canaliculus* powder have established that the lipid fractions (in contrast to earlier work on aqueous fractions) are a rich source of primary and secondary metabolites, and semipurified extracts have shown a measure of anti-inflammatory activity when tested in appropriate model systems.

A reliable source of lipid extract of *Perna canaliculus* and *Mytilus edulis* has become available through the procedure of supercritical fluid extraction (SFE). The lipid rich extract is obtained as a dark yellow-brown viscous oil exhibiting strong ultraviolet absorbing character which is consistent in physical data to oil extracts obtained from earlier large scale solvent extraction procedures. An anti-inflammatory preparation which exhibits significant *in vivo* and *in vitro* activity and which is substantially free of toxic side effects has now been isolated from this lipid extract.

According to one aspect, the present invention provides an anti-inflammatory preparation which comprises a purified, active fraction isolated from a lipid extract of *Perna canaliculus* or *Mytilus edulis* or an active component thereof.

The term "fraction" as used herein means an isolated portion of the initial lipid extract which is identified as a separate region on a chromatogram and may be separated from the remainder of the lipid extract by chromatographic

techniques. The term "active fraction" as used herein means a fraction which exhibits *in vitro* and/or *in vivo* anti-inflammatory activity, which may be demonstrated for example by using the biological assays described herein. The term "active component" as used herein means one or more compounds in an active fraction which individually or together exhibit *in vitro* and/or *in vivo* anti-inflammatory activity.

Preferably, this purified fraction is isolated by high performance liquid chromatography (HPLC) of the lipid extract, using for example reverse phase-HPLC (RP-HPLC) as described in detail hereunder. Preferably also, the fraction is further purified using silver ion-HPLC (Ag-HPLC). The anti-inflammatory preparation of the present invention, may comprise a number of separate active constituent compounds identified by separate peaks on a chromatogram, and accordingly it is to be understood that the term "purified fraction" as used herein extends not only to the fraction including one or more of these active constituent compounds, but also to one or more of the active constituent compounds represented by these separate peaks. These peaks are believed to represent free fatty acids, but may also include fatty acid derivatives within the fraction.

The free fatty acid active component(s) in the anti-inflammatory preparation of this invention may also be converted into a pharmaceutically acceptable ester derivative, for example a methyl or other lower alkyl ester thereof, a pharmaceutically acceptable amide derivative, for example, an amide of the formula -NHR wherein R represents hydrogen or lower alkyl, or a pharmaceutically acceptable salt for example an alkaline or alkaline earth metal salt such as a sodium, potassium or calcium salt thereof. Accordingly, the anti-inflammatory preparation of this invention as broadly described herein includes not only free fatty acids but also the pharmaceutically acceptable esters, amides and salts thereof.

Preferably, the preparation of the invention comprises a purified, active fraction isolated from a lipid extract of *Perna canaliculus*; more preferably, it

comprises a fraction having the characteristics of one of the fractions identified herein as VAR-1, RPFA2, RPFA3, RPFA4, AG5 or AG6, or an active constituent compound thereof. Chromatograms of these fractions, and in some cases molecular ion spectra of constituent compounds therof, are included herein. A particularly preferred fraction is AG5, or an active component thereof.

Further details of the purification of the lipid extract of *Perna canaliculus* to produce the purified active fraction of this invention are disclosed in the Examples below.

The term "purified" as used herein means that the fraction has been isolated or separated from other components of the lipid extract which do not exhibit any or any substantial anti-inflammatory activity. Preferably, the fraction has been purified to the extent that it may be identified by a separate peak or a number of adjacent separate peaks on a chromatogram.

In the work described in detail herein, a major constituent compound of a preferred fraction in accordance with this invention having anti-inflammatory activity has been isolated and, based on GC/MS assignments of double bond positions, identified as 5,11,14,17-eicosatetraenoic acid.

Accordingly, a further aspect of this invention provides the compound, 5,11,14,17-eicosatetraenoic acid, as well as its pharmaceutically acceptable esters, amides and salts. This compound may be prepared in substantially pure form, that is substantially free from other compounds with which it is usually associated in the mussel lipid extract, by chromatographic methods disclosed in detail herein.

In another aspect, the present invention also provides a pharmaceutical composition comprising the anti-inflammatory preparation broadly described above, together with one or more pharmaceutically acceptable carriers and/or diluents. In this aspect, the invention includes a pharmaceutical composition

comprising the compound 5,11,14,17-eicosatetraenoic acid or a pharmaceutically acceptable ester, amide or salt thereof, together with one or more pharmaceutically acceptable carriers and/or diluents.

Such a pharmaceutical composition may, if desired, also include one or more other pharmaceutically active components, for example, one or more known non-steroidal anti-inflammatory agents such as aspirin and indomethacin.

In yet another aspect, the present invention provides a method of treatment of inflammation on an inflammatory condition in an animal, including a human, which comprises administering to the animal an effective amount of an anti-inflammatory preparation as broadly described above. The invention also extends to administration of an effective amount of the compound 5,11,14,17-eicosatetraenoic acid or a pharmaceutically acceptable ester, amide or salt thereof.

In a further aspect, the present invention provides the use of the anti-inflammatory preparation as broadly described above, and in particular the compound 5,11,14,17-eicosatetraenoic acid or a pharmaceutically acceptable ester, amide or salt thereof, in the preparation of a medicament for treatment of inflammation or an inflammatory condition in an animal, including a human.

Preferably, in this aspect the animal treated is a human. In this aspect, the invention provides anti-inflammatory treatment in general, and this includes by way of example, treatment of rheumatoid and osteoarthritis as well as allied inflammatory disorders.

The anti-inflammatory preparation of this invention has been shown to have significant anti-inflammatory activity both *in vitro* in inhibition of leukotriene synthesis and *in vivo* in the adjuvant-induced polyarthritis model in the rat. Since increased leukotriene levels have been associated with the occurrence of asthma and particularly with inflammation of the bronchii associated with accumulation of

neutrophils and the like in the bronchii[5], the anti-inflammatory treatment in accordance with the present invention extends to treatment of asthma as well as to treatment of arthritis and other inflammatory disorders in general.

The magnitude of the effective amount of the anti-inflammatory preparation of this invention will, of course, vary with the group of patients (age, sex, etc.), the nature or the severity of the condition to be treated and the dosage form and route of administration. In general, the daily dose range for use will usually lie within the range of from about 0.1 to about 100 mg per kg body weight of the patient, more preferably from about 0.5 to about 50 mg/kg/day, and even more preferably from about 1 to about 30 mg/kg/day.

Any suitable route of administration may be employed for providing an animal, especially a human, with an effective dosage of the anti-inflammatory preparation of this invention. For example, oral, rectal, vaginal, topical, parenteral, ocular, nasal, sublingual, buccal, intravenous and the like administration may be employed. Dosage forms include tablets, troches, dispersions, suspensions, solutions, capsules, creams, ointments, suppositories, aerosols and the like. Said dosage forms also include implanted slow releasing devices specifically designed for this purpose or other forms of implants modified to additionally act in this fashion.

Other known anti-inflammatory substances, as well as conventional pharmaceutically acceptable carriers, excipients, buffers or diluents, may also be included in the pharmaceutical compositions of this invention. As used herein "pharmaceutically acceptable carriers and/or diluents" include any and all solvents, dispersion media, aqueous solutions, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art and is described by way of example in *Remington's Pharmaceutical Sciences,* 18th Edition, Mack Publishing Company, Pennsylvania, U.S.A.

In practical use, the anti-inflammatory preparation of this invention can be combined as the active ingredient in intimate admixture with a pharmaceutical carrier and/or diluent according to conventional pharmaceutical compounding techniques. The carrier and/or diluent may take a wide variety of forms depending on the form of preparation desired for administration, e.g. oral or parenteral (including intravenous and intra-arterial). In preparing the compositions for oral dosage form, any of the usual pharmaceutical media may be employed, for example, carriers and diluents such as water, glycols, oils, alcohols, flavouring agents, preservatives, colouring agents and the like in the case of oral liquid preparations such as, for example, suspensions, elixirs and solutions; or carriers such as starches, sugars, microcrystalline cellulose, diluents, granulating agents, lubricants, binders, disintegrating agents and the like in the case of oral solid preparations such as, for example, powders, capsules and tablets. Because of their ease of administration, tablets and capsules represent the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are obviously employed. If desired, tablets may be sugar-coated or enteric-coated by standard techniques.

In addition to the common dosage forms set out above, the anti-inflammatory preparation of this invention may also be administered by controlled release means and/or delivery devices.

Pharmaceutical compositions of the present invention suitable for oral or parenteral administration may be presented as discrete units such as capsules, cachets or tablets each containing a predetermined amount of the active ingredient, as a powder or granules or as a solution or a suspension in an aqueous liquid, a non-aqueous liquid, an oil-in-water emulsion or a water-in-oil liquid emulsion. Such compositions may be prepared by any of the methods of pharmacy but all methods include the step of bringing into association the active ingredient with the carrier which constitutes one or more necessary ingredients. In general, the compositions are prepared by uniformly and intimately admixing

the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product into the desired presentation.

It is especially advantageous to formulate compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the human subjects to be treated; each unit containing a predetermined quantity of active ingredient calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier and/or diluent. The specifications for the novel dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active ingredient and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active ingredient for the particular treatment.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Further features of the present invention are more fully described in the following Example(s). It is to be understood, however, that this detailed description is included solely for the purposes of exemplifying the present invention, and should not be understood in any way as a restriction on the broad description of the invention as set out above.

In the accompanying drawings:

Figure 1 shows isocratic reverse phase HPLC of fraction VAR-1.

Figure 2 shows separation of mussel fatty acids (20 mg) by RP-HPLC. Chromatographic conditions: Beckmann Ultrasphere™ C18 column; Flow rate,

1.5 ml/min; Mass detector Evaporator set value (ESV) +60; gas pressure, 14 psi; The relative time of elution of the constituent fatty acids in the mixture are indicated.

Figures 3, 4 and 5 show GC chromatograms of the fractions RPFA-2, RPFA-3 and RPFA-4, respectively.

Figure 6 shows separation of mussel fatty acid methyl esters, FAME, (2 mg) by Ag-HPLC. Chromatographic conditions: Chrompack, Chromsphere™ C18 column; Flow rate, 2.0 ml/min; Mass detector Evaporator set value (ESV) +40; gas pressure, 14 psi. The relative time of elution of the constituent FAMEs in the mixture are indicated.

Figures 7 and 8 show GC chromatograms of the fractions AG5 and AG6, respectively.

Figures 9, 10, 11, 12, 13 and 14 show molecular ion spectra of fractions AG5 (C18:4), AG5 (C19:4), AG5 (C20:4), AG5 (C20:4), RPFA3 (C20:5) and RPFA3 (C21:5), respectively.

EXAMPLE 1

I PREPARATION OF LIPID EXTRACT

1.1 Raw Material

The green lipped mussel *(Perna canaliculus)* is harvested on the south coast of New Zealand at which time the total mussel is stabilised with tartaric acid. Freeze drying results in a dry powder of pulverised form.

1.2 Extraction of Lipids

The technique of supercritical fluid extraction (SFE) is utilised to extract the biologically active lipids from the crude mussel powder.

Cryogenic fluid CO_2 is used as the extracting medium. The CO_2 is expanded to atmospheric pressure and the extract is presented as a concentrated oil. The powder yields 3-3.5% of oil.

1.3 Profile of the crude oil

The extractable oil is orange amber in colour and is a viscous liquid at ambient temperature. The extract is stored below 4°C and is handled in a nitrogen atmosphere. The crude oil shows strong UV activity and is protected from light to minimise the polymerisation of double bond components.

II BIOLOGICAL ASSAYS.

2.1 *In vivo* Mycobacterial adjuvant-induced polyarthritis.

Induction of adjuvant arthritis was made in the following manner by a modification of the method of Winter, Risely and Nuss[3] according to McColl *et al.*[6]. A complete Freund's adjuvant containing heat killed dry *Mycobacterium tuberculosis* suspended in an oily vehicle (squalane) at a final concentration of 10 mg/ml was injected intradermally (0.05ml) into the tail of male Dark Agouti or Long Evans rats weighing between 200 to 250g. The injected tail became inflamed (increase in volume) and reached maximal size within three to five days (primary lesion). Assessment of the inflammation was made 2 weeks after tail injection. Secondary lesions of adjuvant arthritis occurred after a delay of approximately 10 days and were characterised by inflammation of non-injected sites and a decrease in body weight gain. Vehicle treated controls and adjuvant controls served as reference groups to determine body weight and paw thickness changes in experimental groups.

Doses of the anti-inflammatory preparation were administered subcutaneously on days 10-13 and inhibition of paw swelling was evaluated on day 14.

Four variables were used to quantify the severity of the disease

1. rear paw swelling between the dorsum of the sole of the foot measured at the midpoint between the posterior border of the calcaneum and the 5th metatarophalangeal joint (using digital calipers)
2. widest diameter of swelling in the tail in the region of injection (using digital calipers)
3. weight change and
4. disease activity (total of 0-4 points) determined as follows:

For each rat paw (0-4) points where

0 = no localised articular lesion or swelling
1 = localised articular lesions or ankle swelling
2 = localised articular lesions and ankle swelling
3 = moderate generalised ankle and foot swelling and
4 = gross generalised ankle and foot swelling.

For each forepaw (0-3) points where

0 = no localised articular lesion or swelling
1 = localised articular lesions or swelling
2 = localised articular lesions and wrist swelling
3 = gross generalised paw and wrist swelling.

All assessment of disease activity were undertaken by an observer unaware of the nature of the anti-inflammatory preparations.

2.2. *In vitro* inhibition of LTB_4 and its 6-trans isomers in PMNLs.

Reverse phase HPLC assay is now established as a reliable measure of leukotriene production in human polymorphonuclear leukocytes (PMNLs) and the lowering of measured levels of 6-LTB_4, 12epi, 6t-LTB_4 are formed from the non-enzymic hydrolysis of LTA_4 while LTB_4 is produced from LTA_4.

The anti-inflammatory preparation has been examined for its effect on leukotriene production after incubation in PMNL preparation. The levels of LTB_4, 6t-LTB_4 and 12epi, 6t-LTB_4 produced were measured by the method of reverse phase HPLC according to Cleland *et al.* [6].

III RESULTS

2.1 Initial results from biological assay.

The first preparative isolation of the crude oil was achieved by normal phase HPLC with UV detection at 280nm. This method afforded the fractionation of five fractions which were coded as 1A to 5A. The polar fractions (3A, 4A, 5A) were derivatised to the fatty acid methyl esters (3ES, 4ES, 5ES) and subjected to the *in vitro* test for anti-inflammatory activity by inhibition of leukotriene synthesis described above. The assay demonstrated significant inhibition (Table 1a). These results are comparable to the principal results from the animal model using the adjuvant-induced polyarthritis model in the rat also described above (Table 1b). These two independent assays lead to the conclusion that fractions 3 and 4 are responsible for the biological activity.

3.2 Preliminary confirmation by TLC analysis.

The oil was dissolved in dichloromethane and spotted onto precoated silica gel TLC plate. The eluting solvent mixture was hexane, ether, acetic acid. Visualisation of the components was achieved by spraying the plate with a 10% copper sulphate in an 8% phosphoric acid solution. The components appear as dark spots on a white background when heated to 110°C.

A preparative thin layer method (PLC) was used to isolate the polar material (code 3PLC, *R, band* 0.1-0.5) from the lipophilic extract. The 3PLC sample was again screened for activity against the neutrophil, leukotriene synthesis inhibition *in vitro* model and the chronic polyarthritis

in vivo animal model. The results for the *in vitro* assay are summarised in Table 2a and support the *in vivo* results presented in Table 2b. Polar band 3PLC has shown a significant inhibitory activity in both assays.

3.3 Preliminary fractionation of crude mussel oil.

Flash chromatography by the method of Still *et al.*[8] on Silica G60 (230-400 mesh) using low pressure column chromatography has been utilised to provide a rapid preliminary large scale separation of individual lipid fraction. This classical method of purification was used as an alternative to HPLC to isolate the active polar material (3PLC) for further analysis. The lipophilic material is eluted with hexane and the polar fraction is isolated after step-wise increases with ether.

The 3PLC fraction shows UV activity with band intensity at 320, 295, 285, 275, 265 and 235nm, indicating unsaturation with possible conjugation. Further investigations will enable use of disposable extraction cartridges packed with silica to isolate the active lipids (3PLC). On the basis of the initial screening, the polar component (5% ether fraction) compared favourably to 3PLC.

3.4 Separation of Lipids by HPLC.

(i) Narp-HPLC (Non aqueous reverse phase).

Narp-HPLC has been utilised to isolate the lipids by carbon number in a multi-step gradient method. The HPLC operating parameters are listed below:

Chromatograph	Applied Biosystems Model 151A
Column	Beckman Ultrasphere ODS 80 Å, 5μm 25 cm x 10 mm (I.D.) C/N 235328
Flow	1 ml/min

Gradient — Multi-step gradient method (see below).

Detector — ACS (Applied Chromatography Systems) Evaporative Light Scattering detector Model 750/14

Multi-Step Gradient

Segment	Segment length (Mins)	% Acetonitrile	% Dichloro-methane
0	15	80	20
1	10	70	30
2	10	55	45
3	15	45	55
4	10	0	100
5	5	0	100
6	10	80	20

The lipid fraction with chemical characteristics similar to 3PLC, was injected onto the column and four fractions were collected based on their elution time and coded VAR-1, VAR-2, VAR-3 and VAR-4, respectively.

The separation by Narp-HPLC is a time-windows technique and the following summarises collection character.

NARP time-windows collection.

Code	Time (min)
VAR-1	11-20
VAR-2	20-27
VAR-3	33-47
VAR-4	47-59

The VAR fractions have been tested by the leukotriene assay and the results are summarised in Table 3a. The results using the chronic inflammation assay are shown in Table 3b. Both tests have been consistent in showing that VAR-1 has retained significant biological activity at a reduced dosage level of 10 mg/kg body weight in comparison to the earlier assays of 50 and 30 mg/kg body weight. This indicates that purification of the bioactive material has taken place with significant increase in specific activity. The parameters which have been measured in Table 3b indicate that fraction VAR-1 has retained anti-inflammatory activity and significantly outperforms all other extracts so far examined.

A TLC analysis of VAR-1 using hexane/ether/acetic acid of proportions (80:20:2) has an R_f 0.38 by visualisation with the copper sulphate acid spray.

(ii) Reverse phase HPLC

The active fraction VAR-1 (identified by the time-window 11-20 minutes) has been further chromatographed using isocratic aqueous reverse phase liquid chromatography. The chromatogram (Figure 1) indicates the separation of four main compounds of similar polarity associated with eight minor constituents within a time window of 2-15 minutes. The analytical separation when compared to known standards (palmitic and myristic acid) indicate that the sample VAR-1 is a mixture of fatty acids. The operating conditions of this analysis are summarised as follows:

Chromatograph	Applied Biosystems Model 151A
Column	Waters Deltapak C18 100A 5μm 15 cm x 3.9 mm (I.D.) C/N 11795
Flow	0.7 ml/min

Mobile phase	Acetonitrile 82 Water 18 Acetic acid 0.2
Detector	ACS (Applied Chromatography Systems) Evaporative Light Scattering detector Model 754/14.

Table 1a % Inhibition of LTB_4 and its 6-trans isomers.

Compound	Dilution	% Inhibition		
		LTB_4	Isomer 1	Isomer 2
3ES	stock (15.2 mg/2.5 ml)	100	100	100
	1:2	100	100	100
	1:4	75	86	84
	1:10	66	81	78
4ES	stock (16.6 mg/2.5 ml)	100	100	100
	1:2	100	100	100
	1:4	75	83	85
	1:10	43	63	67

Table 1b Chronic inflammation assay

Sample Code	Expt. No.	Dose (mg/kg)	% Inhibition of paw swelling
3es	#1278	50	93
3es	#1281	30	85
4es	#1278	50	58

Table 2a % Inhibition of LTB_4 and its 6-trans isomers.

Compound	Dilution	% Inhibition			
		Isomer 1	2	LTB_4	5HETE
3PLC (6.5 mg/ml)	1:100*		89	6	83
	1:1000	71	72	21	40
	1:10000	30	31	2	11

* with the higher concentrations of the oil there was considerable interference of the solvent front with the internal standard, so that quantitation was difficult in the HPLC analysis.

Table 2b Chronic inflammation assay.

Sample Code	Expt. No.	Dose (mg/kg)	% Inhibition of paw swelling
3PLC	#1	30	63
3PLC	#2	30	58

Table 3a % Inhibition of LTB_4 and its 6-trans isomers.

Compound	Dilution	% Inhibition		
		LTB_4	Isomer 1	Isomer 2
VAR-1 (4.5 mg/0.5 ml)	1:100	85	100	100
	1:1000	35	67	62
	1:10000	11	7	17
VAR-2 (4 mg/0.5 ml)	1:100	91	100	100
	1:1000	16	35	38
	1:10000	11	10	8
VAR-3 (11.8 mg/0.5 ml)	1:100	63	71	62
	1:1000	8	8	12
	1:10000	7	0	0
VAR-4 (5.2 mg/ml)	1:100	51	51	44
	1:1000	6	0	0
	1:10000	14	18	20

Table 3b Chronic inflammation assay.

Sample Code	Expt.	Dose (mg/kg)	% Inhibition paw swelling	% Inhibition, Inflammatory rating	% Inhibition of weight loss
VAR-1	#14-6	10	88	48	79
VAR-2	#14-6	10	0	0	0
VAR-3	#14-6	10	46	22	0
VAR-4	#14-6	10	0	0	0

EXAMPLE 2

A. MATERIALS AND METHODS

1.1 Pilot Scale SFE

Extraction of total lipids in freeze-dried mussel powder, *Perna Canaliculus* was performed on a pilot scale SFE unit undertaken at the Food Research Institute (Department of Agriculture, Werribee, Vic., Australia).

Instrumentation

Extractions were performed on a pilot scale extraction unit consisting of five basic sub-units (Distillers MG Limited., England, UK). The five basic units comprise: Carbon dioxide supply, Solids extraction, Primary Separation, Evaporation and tailing units.

The carbon dioxide supply unit consists of two CO_2 cylinders connected in parallel and placed on a weighing scale for recharging when appropriate. The extraction unit can be supplied with liquid SC-CO_2 and SC-CO_2. For this work the plant was operated using SC-CO_2. Solid material was placed in the leaching column and the primary separator facilitates separation of extracted material by reduction of pressure (which allows extract to settle), adsorption or liquid extraction. The fluid extract was passed into the evaporation unit to evaporate the CO_2 by the use of internal heating tubes. The vapour may contain volatiles

and thus it is subsequently passed to the tailing column to be scrubbed by pure liquid CO_2. The tailing unit traps the gaseous CO_2 from the evaporator unit and returns the volatile components to the evaporator.

Pilot plant Extraction procedure

Mussel powder (300 g) was charged to the extraction unit (leaching column). SC–CO_2 was delivered at a flow rate of 3.0 kg/h. for two hours per extraction. Extractor temperature was set at 40°C and the extractor pressure at 310 bar (4,500 psi). The evaporator temperature was held constant at 40°C. The mussel lipid extracts were stored under nitrogen at -10°C in amber glass sealed containers.

1.2 Column Chromatography

Fractionation of the mussel lipids was performed by open column flash chromatography using silica gel[8]. Silica gel Kieselger 60 (E. Merck, Darmstadt, Germany) particle size 230-400 mesh was weighed and a slurry of the silica in dichloromethane (AR) was prepared. The mussel lipids (1 g per 10 g of silica adsorbent) in dichloromethane was carefully applied to the column.. Triglycerides and cholesterol esters were eluted in 2 bed volumes (BV) of dichloromethane. Separation of the mussel classes was carried out by stepwise gradient elution by increasing the amount of methyl *tert*-butyl ether (MTBE) in the hexane. The isolation of the free fatty acid (FA) class was achieved with 79.9% hexane; 20% MTBE; 0.1% acetic acid (4 BV). The FA fraction was collected and were subsequently reduced in volume to an oily residue (24 %, w/w) by rotary evaporation. The residues were transferred to vials and further dried under a stream of nitrogen until constant in weight. Purity of the free fatty acid fraction was verified by TLC on aluminium-backed silica plates.

1.3 Thin Layer Chromatography (TLC)

Materials

A lipid mixture (cholesterol ester CE, triglyceride TG, fatty acid FA, cholesterol C and phospholipid PL) was purchased from Nu-Chek-Prep Inc (Elysian, MO, USA). Distearin and monopalmitin were also obtain from Nu-Chek-

Prep Inc. Analytical grade hexane (95%) was from E. Merck, (Darmstadt Germany), and glacial acetic acid from Ajax Chemicals Pty. Ltd. (Sydney, Australia).

HPTLC developing system

HPTLC aluminium sheets coated with silica gel 60 F 254 (E. Merck, Darmstadt, Germany) were cut to an appropriate size (10 cm x 20 cm) and used without prewashing. Mussel fractions and the lipid standards were dissolved in dichloromethane. The direct sample application method was performed using a micropipette (Becton-Dickinson and Co., Parsippany, NJ, USA) and compared to the standard. The chromatographic separation was carried out in hexane-diethyl ether-acetic acid (80+20+1, v/v/v). Bands were identified after spraying with 10% copper sulphate in 8% phosphoric acid reagent and charring at 110°C for 20 min. (Bitman and Wood, 1982).

1.4 High Performance Liquid Chromatography (HPLC)

Instrumentation

An Applied Biosystems chromatograph (Model 150A) consisted of a Model 1400A dual pump solvent delivery system (Applied Biosystems, Foster City, Ca, USA) and a Model 1783A absorbance controller unit. A WISP 712 Waters Intelligent Sample Processor and a mass detector, Model 750/14, ACS (Applied Chromatography Systems Ltd., Macclesfield, Cheshire, UK) coupled to high purity N_2 gas (Linde Gas Pty. Ltd., Sydney, Australia). The inclusion of a splitter (consisting of a stainless steel block with 3 apertures) permitted preparative collection of the mussel fatty acids. The column was diverted to a sample collector (Gilson Instruments, Model 201, Villiers, LE, BEL, IR).

1.4.1 Isolation by RP-HPLC

An Ultrasphere™ C18, 80 Å, 5 μm, 25 cm x 10 mm (I.D.) column (Beckmann) with a 1 cm x 0.45 mm (I.D.) 5μm, C18 scavenger column (Activon Scientific Products, Thornleigh, NSW, Australia) inserted in front of the column

were used to separate the mussel fatty acids. The mass detector was set to 14 psi and the evaporator set value was +60.

Sample Preparation

The mussel fatty acids were diluted to 500 μg/ml in 1mL AcCN and THF (1:1, v/v) and an aliquot of 45 μL was injected on column. The samples were filtered before column injection through a 0.45 μm nylon syringe filter (Activon Scientific Products, Thornleigh, Australia). The above preparations were stored at -10°C during the course of this work.

Elution system for RP-HPLC

Table 4. Elution profile for fatty acid lipid class separation on Ultrasphere™ C18 reverse phase.

Segment	Time (min.)	%Water	%Acetonitrile	%Methanol
0	0	13	87	0
1	35	13	87	0
2	40	0	100	0
3	42	0	0	100
4	57	0	50	50
5	59	13	87	0

From Table 4, an initial solvent composition consisting of 13% water - 87% acetonitrile (AcCN) was held for 35 mins to isolate the individual species according to their carbon number and degree of unsaturation. At 40 mins., the final solvent composition was 100% AcCN (segment 2) by linear solvent strength gradient elution of AcCN into 13% water-AcCN. Segment 3 permitted elution of long chained fatty acids. The column was regenerated (Segment 4-5) as preparation for the next sample. The flow rate was maintained at 1.5 mL/min.

1.4.2 Silver ion chromatography

Chromatographic system

A Chromspher™ column, 10 μm, 5 μm, 25 cm × 10mm (ID) (Chrompack, Netherlands). A binary gradient was prepared with solvent reservoir A; dichloroethane-dichloromethane 1:1 (v/v) and solvent reservoir B; dichloroethane-dichloromethane-methanol-acetonitrile 4/4/1/1, (v/v/v/v). The ACS mass detector

parameters were set to +40 and the gas pressure 14 psi. The flow rate was maintained at 2.0 mL/min.

Table 5. Binary elution profile of separation of fatty acid methyl esters by silver chromatography.

Segment	Time (min.)	%A	%B
0	0	70	30
1	31	8	92
2	35	8	92
3	43	0	100
4	53	0	100
5	55	70	30

Preparation of mussel fatty acid methyl esters (FAME)

Mussel fatty acids were isolated by RP-HPLC and methylated by dissolving in 12.5% BF_3 methanol[9]. After the mixture was refluxed for 1 h. at 70°C, heptane solvent (20mL) was added and refuxed for a further 15 min. The organic layer is washed first with saturated sodium chloride solution (15mL). Methyl *tert*-butyl ether (2×50 mL) was added and the extract was washed with water (2×20 mL). The FAME mixture was dried over anhydrous sodium sulphate powder, filtered, concentrated and chromatographed on a silica gel G60 column previously conditioned with hexane. Elution of mussel FA methyl esters was achieved in 10% MTBE in hexane (v/v). TLC (silica gel) analysis verified the clean mixture after visualisation of the products was achieved by spraying with Usui's reagent which consisted of 5 g of phosphomolybdic acid (PMA) dissolved in 100 mL acetic acid and 7.5 mL sulphuric acid and charring the plate to 110°C. All solvents were HPLC grade: sodium sulphate, PMA and other acid reagents were obtained from Ajax Chemicals Pty. Ltd. (Sydney, Australia).

1.4.3 Profiling of HPLC fractions by high resolution gas chromatography

An Shimadzu GC (Model GC-17A) with a flame ionisation detector (set at 260°C) was linked to a Shimadzu Chromatopac integrator. A BPX70 cyanopropyl polysiphenylene-siloxane capillary column, 50m × 0.22 mm (I.D) (SGE, Austin, Texas) with helium as the carrier was used to analyse the fatty acid methyl esters

(standardised with C17:0 FAME). The system was temperature programmed as follows:

1 min.	▸ ▸ ▸ ▸	4 mins.	▸ ▸ ▸ ▸	t end 37 mins.
T(120°C)	5°C/min	T(170°C)	10°C/min	T(220°C)

Injector temperature was set at 260°C and the linear velocity of the helium gas was 20 cm/min.

1.5 Gas Chromatography Mass Spectrometry

GC analysis was carried out on a Hewlett-Packard 5890 GC equipped with an HP1 column (12 mm x 0.2 mm x 0.33 μm) with an inlet pressure of 5 psi. The oven temperature was held at 75°C for 2 min. then heated at 30°C/min. to 300°C with a final holding time of 9 min. Injections were spitless. An injector temperature of 200°C and a detector temperature of 300°C were used.

Electron impact gas chromatography mass spectroscopy (EI GC/MS) was performed on a Hewlett Packard 5890 GC (Finnigan MAT INCOS 50 MS). Chromatographic conditions were identical to those described above. The interface temperature was 280°C, the injector 250°C and the ion source temperature was 180°C. A scan range of 50 to 500 Da at 0.6 sec/scan was used.

Positive ion chemical ionisation (PICI) GC/MS was performed on a Hewlett Packard 5890 GC (Finnigan MAT TSQ-80 MS). Conditions were the same as those described above.

B. RESULTS

2.1 Pilot scale SFE

Large scale runs were carried out at the Food Research Institute of Victoria in order to obtain suitable quantities for further analyses. A maximum yield of 4.76% was obtained at the maximum extraction temperature and pressure (345

bar, 40°C-70°C). The extract was orange-amber in colour with a viscous waxy appearance. The products from each run were collected and stored at -10°C.

2.2 Separation by lipid class

Flash Column Chromatography

The separation of the mussel extract into lipid class was facilitated by open column flash chromatography. Preliminary separation of lipids according to polarity was achieved on silica gel. Cholesterol esters and triglycerides were eluted in dichloromethane solvent. The free fatty acids were eluted in hexane-MTBE (80 + 10, v/v).

2.3 TLC

Ascending one dimensional TLC was used to screen the fractions from the preparative column chromatography of the crude mussel extract. Separation on silica gel plates resolved the lipid classes. Rapid analysis occurred following development in 80+20+1 (v/v/v) hexane-diethyl ether-acetic acid. The components are resolved into black spots on a white background by spraying with a copper sulphate reagent upon drying in a 110°C oven for 20 minutes. The free fatty acid components was verified at R_f equivalent to 0.34.

2.4 Chromatography

2.4.1 RP-HPLC

A standard octadecasilyl phase (ODS) has been employed to achieve separation of molecular fatty acid species. A typical chromatogram of the separation achieved by RP-HPLC is shown in Figure 2.

In vitro analysis of fatty acid fractions (RP1-RP8) purified by RP-HPLC.

The RP fractions were concentrated by rotary evaporation at 40°C and reconstituted in methanol. The fractions were screened by an *in vitro* model of inflammation by leukotriene synthesis[10]. Components RPFA-2, RPFA-3 and RPFA-4 indicated significant biological activity (denoted by an asterix *, in Figure 2 and Table 6).

Table 6 Inhibition of LTB_4 and 6-trans isomers

Code	Dilution	% Inhibition			
		Isomer 1	Isomer 2	LTB_4	5-HETE
RPFA-1	1:100	13	13	2	0
	1:1000	0	0	3	0
	1:10000	8	10	4	4
RPFA-2*[1]	1:100	100	100	100	100
	1:1000	24	40	47	3
	1:10000	12	12	20	0
RPFA-3*[1]	1:100	94	100	79	100
	1:1000	29	43	13	12
	1:10000	12	26	8	7
RPFA-4*[1]	1:100	100	100	100	100
	1:1000	43	58	25	23
	1:10000	20	29	13	6
RPFA-5	1:100	97	100	89	83
	1:1000	25	43	13	0
	1:10000	19	36	6	0
RPFA-6	1:100	22	33	16	10
	1:1000	0	0	0	
	1:10000	0	0	0	0
RPFA-7	1:100	44	59	22	22
	1:1000	0	6	0	0
	1:10000	8	19	6	5
RPFA-8	1:100	26	43	14	3
	1:1000	11	12	14	0
	1:10000	0	0	8	0
RPFA-9	1:100	0	0	17	0
	1:1000	0	0	0	0
	1:10000	0	0	0	38
RPFA-10[2]	1:100	89	100	100	89
	1:1000	0	0	0	0
	1:10000	0	0	0	0

[1] RPFA samples have been further analysed by high resolution GC.

[2] parent compound i.e. total free fatty acids isolated by open column chromatography (see materails and methods 1.2).

Profiling of RP fractions by high resolution Gas Chromatography

The RP fractions were profiled by high resolution GC by the conditions described in Section 1.4.3. Known fatty acids were identified by direct comparisons of their retention times to those of a commercial standard (Nu-Chek-Prep Inc. (Elysian, MO, USA). The GC chromatograms of RPFA-2, RPFA-3 and RPFA-4 as their methyl esters are shown in Figures 3, 4 and 5, respectively. The peak corresponding to 26.93 mins. is common to RPFA-3 and RPFA-4 and was deemed to be contain the bio-active constituent. Focus was given to fraction RPFA-3 which is rich in component at 26.93 mins. and has also exhibited *in vitro* activity.

2.4.2 Ag-HPLC

Source of material

Preparative amounts of the mussel free fatty acids were subjected to RP-HPLC. Components RPFA2 to RPFA4 were collected by their respective time windows (Table 7). Fraction RPFA -3 was prepared for silver ion chromatography by formation of the methyl ester derivative.

Table 7. Time windows for collection of RP fractions 2-4 isolated by RP-HPLC.

Sample	Time window, t (min.)	
	t_{start}	t_{end}
RPFA-2	20.8	24.4
RPFA-3	24.4	30.8
RPFA-4	30.8	40.4

The preparative separation achieved by AG-HPLC is displayed in Figure 6. The separation is primarily based on the number of double bonds present; secondary effects are possible due to presence of carbonyl group.

In vitro analysis of fractions after silver ion chromatography

The active isolate, RPFA3 was further purified by AG-HPLC using a binary gradient program (Section 1.4.2). The fractions from silver ion chromatography (see Figure 6) were separated into eight constituents; the major component at 46.9 min. corresponding to docosahexaenoic acid (DHA). Interbands AG2 and

AG4 were not included in the *in vitro* assay due to scarcity of material within these time windows.

Table 8 %Inhibition of LTB_4 and 6-trans isomers.

Code	Time window t, (min) t_{start} t_{end}	% inhibition (% of control)				
		dilution	isomer 1	isomer 2	LTB4	5-HETE
AG1	4.0 - 10.0	1:100	41	34	9	29
		1:1000	28	27	29	23
		1:10000	25	26	30	23
AG3	15.5 - 21.0	1:100	65	59	51	54
		1:1000	3	2	0	
		1:10000	10	11	0	20
AG5	24.0 - 26.0	1:100	82	70	64	71
		1:1000	0	0	4	10
		1:10000	0	0	0	6
AG6	26.0 - 33.7	1:100	48	46	47	44
		1:1000	0	0	11	8
		1:10000	0	0	2	11
AG7	33.7 - 39.9	1:100	25	21	3	40
		1:1000	0	0	1	4
		1:10000	0	0	0	0
AG8	39.9 - 56.9	1:100	74	77	91	72
		1:1000	41	35	37	23
		1:10000	0	0	15	14

From Table 8, anti-inflammatory activity is exhibited in fraction AG5 and AG8 (1:100 dilution). At the low concentrations of 1:1000 and 1:10000, AG5 exhibited low inhibitory activity due to experimental insolubility in the test medium. The time elution of AG5 and AG6 is comparable to the retention of authentic standards containing tetraene and pentaene components.

Profiling of RP fractions by high resolution Gas Chromatography

Identification of the fatty acid composition of AG5 indicated the enhanced presence of the unknown peak at retention 27.03 mins. (Figures 7 and 8). The presence of this peak in AG6 may be due to carryover effects from AG5 during the Ag-HPLC separation. Structural elucidation of the fractions has been determined by high resolution and GC/MS.

2.5 GC/MS

The major constituents of the active fraction, AG5 were identified by GC/MS (Figures 9 to 14) and assignments of double bond positions of the methyl esters are given in Table 9. A commercial mixture of standard FAMEs was used with an internal standard (C17:0) to correlate retention times with bond unsaturation. These standards were also used to assist in double bond location for structural elucidation of the active constituents.

Table 6. Summary of assignment of fraction AG5 and RPFA-3.

Sample	Component retention from GC (mins)	Chemical assignment		GCMS Figure
		Carbon number	Position of double bonds	
AG5	23.469	18:4	4,9,12,15 and/or 6,9,12,15	9
AG5	24.476	19:4	5,10,13,16 and/or 6,10,13,16	10
AG5	26.001[3]	20:4	5,8,11,14	11
AG5	27.032	20:4	5,11,14,17	12
RPFA-3	27.542[4]	20:5	5,8,11,14,17	13
RPFA-3	29.928	21:5	5,8,11,15,18	14

[3] Peak at 26.001 mins. is known and has been identified as arachidonic acid methyl ester.

[4] Peak at 27.542 mins is known and has been identified as eicosapentaenoic acid methyl ester (EPA).

Comparison of the electron impact spectra of the methyl esters of arachidonic acid (5,8,11,14-eicosatetraenoic acid) and EPA (5,8,11,14,17-eicosapentaenoic acid) with that of the methyl ester of the fatty acid at retention peak 27.03 mins. supports the assignment of this fatty acid as 5,11,14,17-eicosatetraenoic acid.

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CLAIMS:

1. An anti-inflammatory preparation which comprises a purified, active fraction isolated from a lipid extract of *Perna canaliculus* or *Mytilus edulis*, or an active component thereof.

2. A preparation according to claim 1, wherein said active fraction or active component is isolated by high performance liquid chromatography (HPLC).

3. A preparation according to claim 2, wherein said isolation is by reverse phase-HPLC, optionally followed by silver ion-HPLC.

4. A preparation according to claim 1, wherein said active fraction is a fraction having the characteristics of one of the fractions identified as VAR-1, RPFA-2, RPFA-3, RPFA-4, AG5 and AG6, or an active constituent compound thereof.

5. A preparation according to claim 4 wherein said active fraction has the characteristics of the fraction identified as AG5, or an active constituent compound thereof.

6. A preparation according to claim 1, comprising free fatty acids, or pharmaceutically acceptable esters, amides or salts thereof.

7. A preparation according to claim 6, wherein said esters are selected from methyl and other lower alkyl esters, said amides are selected from compounds having a -NHR moiety wherein R represents hydrogen or lower alkyl, and said salts are selected from sodium, potassium, calcium and other alkaline and alkaline earth metal salts.

8. A pharmaceutical composition comprising an anti-inflammatory preparation of any of claims 1 to 7, together with one or more pharmaceutically acceptable carriers and/or diluents.

9. A method of treatment of inflammation or an inflammatory condition in an animal, including a human, which comprises administering to the animal an effective amount of an anti-inflammatory preparation of any of claims 1 to 7.

10. A method according to claim 9, wherein the inflammatory condition is arthritis, including rheumatoid or osteoarthritis.

11. A method according to claim 9, wherein the inflammatory condition is asthma.

12. Use of an anti-inflammatory preparation of any of claims 1 to 7 in the preparation of a medicament for treatment of inflammation or an inflammatory condition in an animal, including a human.

13. The compound 5,11,14,17-eicosatetraenoic acid, in substantially pure form.

14. The compound 5,11,14,17-eicosatetraenoic acid, or pharmaceutically acceptable esters, amides or salts thereof.

15. A compound according to claim 14, wherein said esters are selected from methyl and other lower alkyl esters, said amides are selected from compounds having a -NHR moiety wherein R represents hydrogen or lower alkyl, and said salts are selected from sodium, potassium, calcium and other alkaline and alkaline earth metal salts.

16. A pharmaceutical composition comprising a compound of any of claims 13 to 15, together with one or more pharmaceutically acceptable carriers and/or diluents.

17. A method of treatment of inflammation or an inflammatory condition in an animal, including a human, which comprises administering to the animal an effective amount of a compound of any of claims 13 to 15.

18. A method according to claim 17, wherein the inflammatory condition is arthritis, including rheumatoid or osteoarthritis.

19. A method according to claim 17, wherein the inflammatory condition is asthma.

20. Use of a compound of any of claims 13 to 15 in the preparation of a medicament for treatment of inflammation or an inflammatory condition in an animal, including a human.

FIGURE 1

HPLC profile of fraction VAR-1

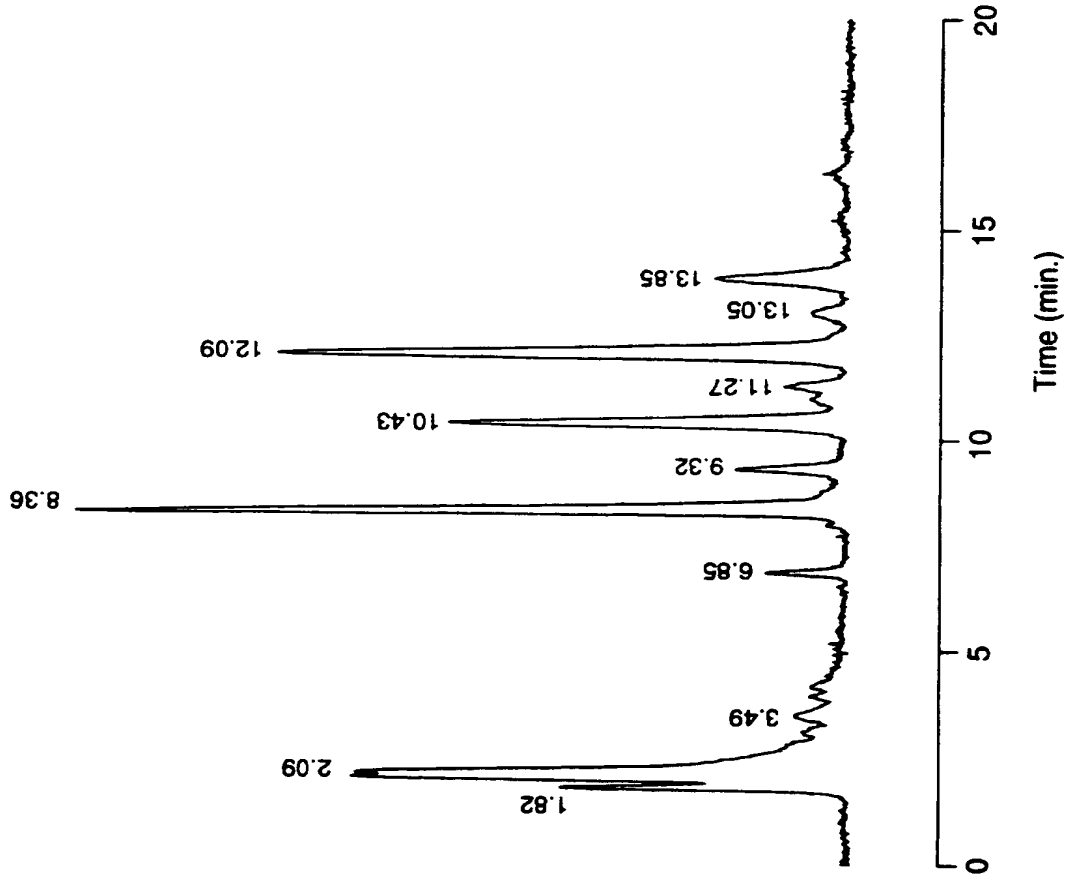


FIGURE 2

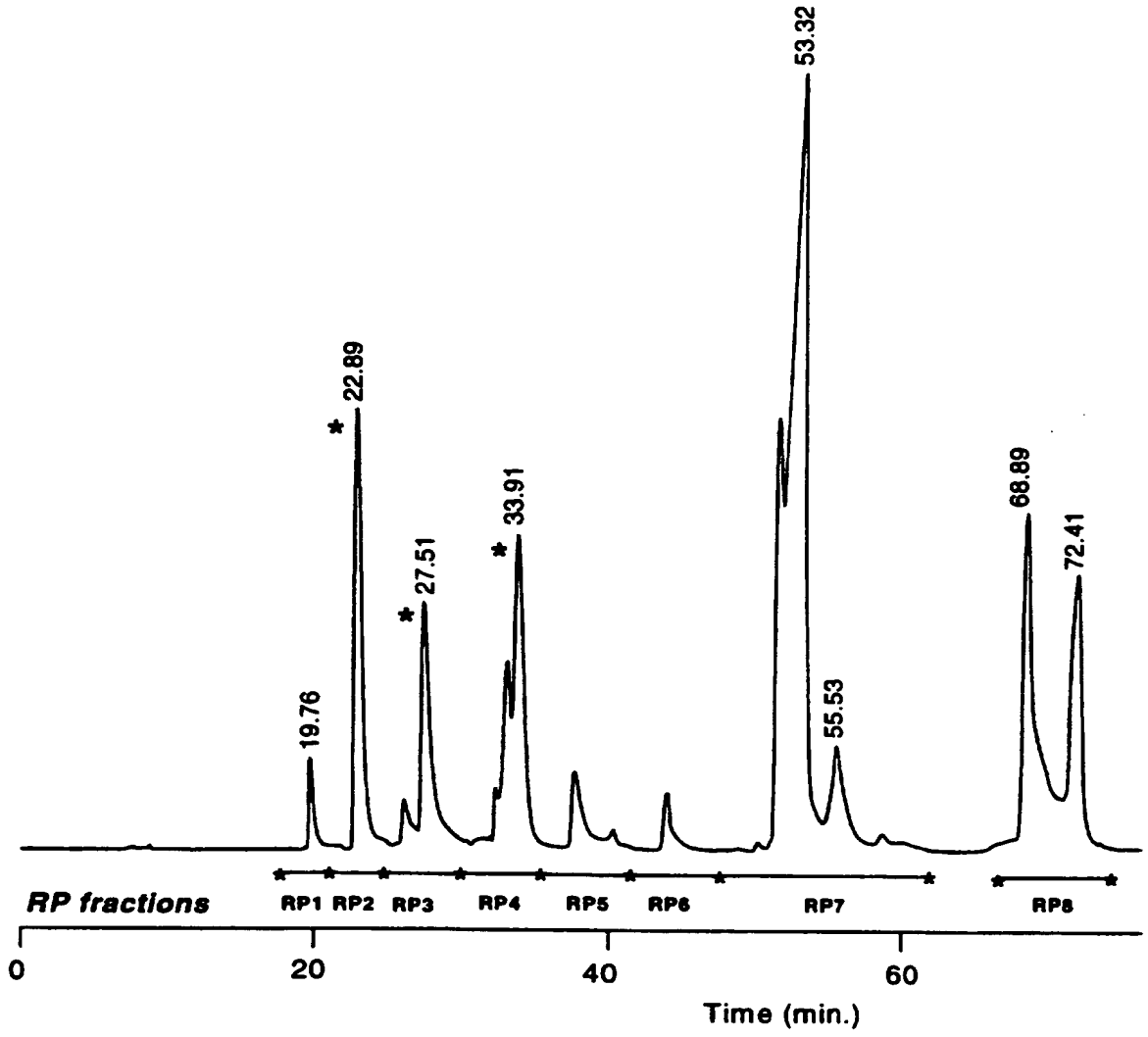


FIGURE 3

GC profile of RPFA-2 (RP-HPLC time window 20.8-24.4 mins)

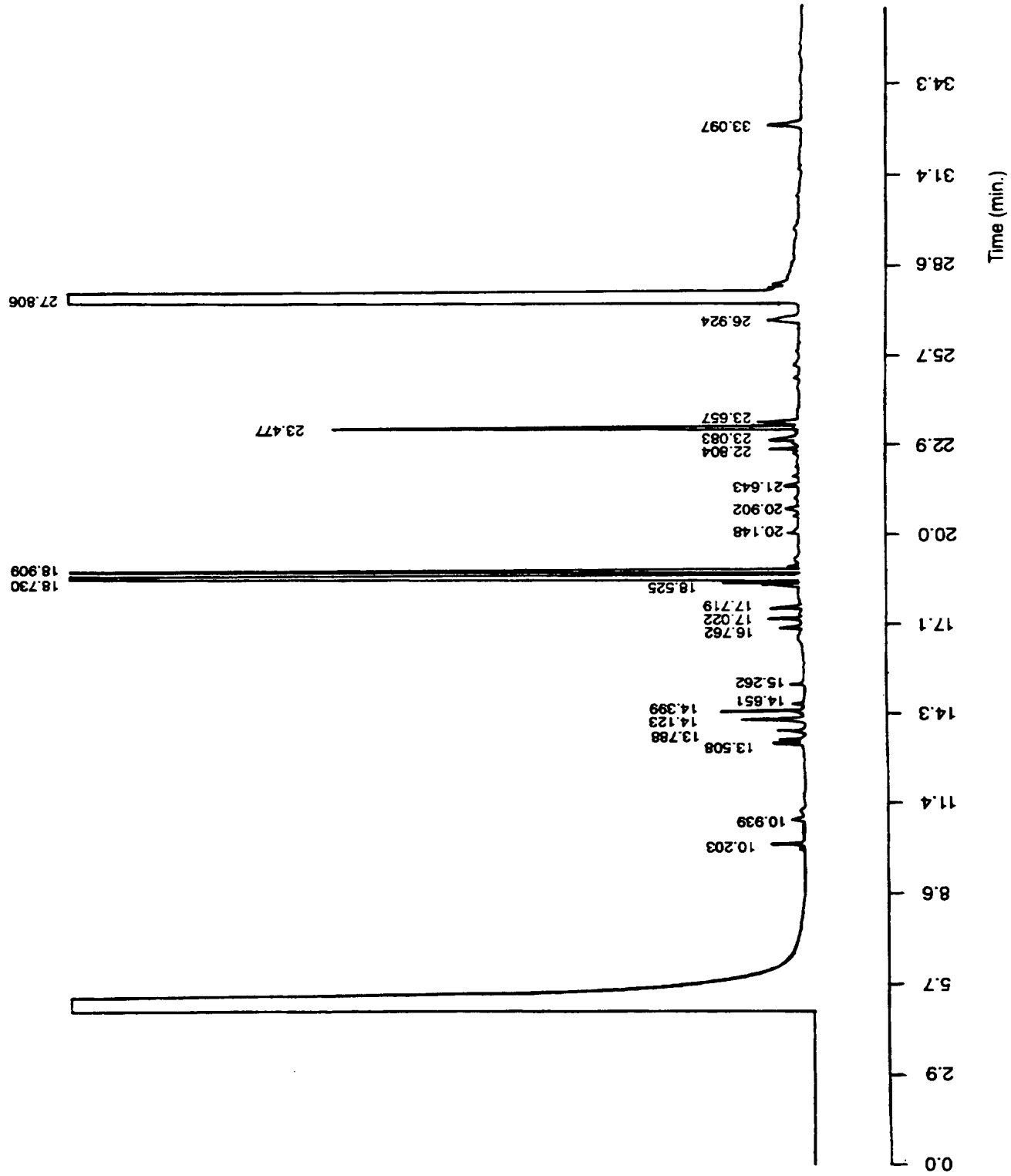


FIGURE 4

GC profile of RPFA-3 (RP-HPLC time window 24.4-30.8 mins)

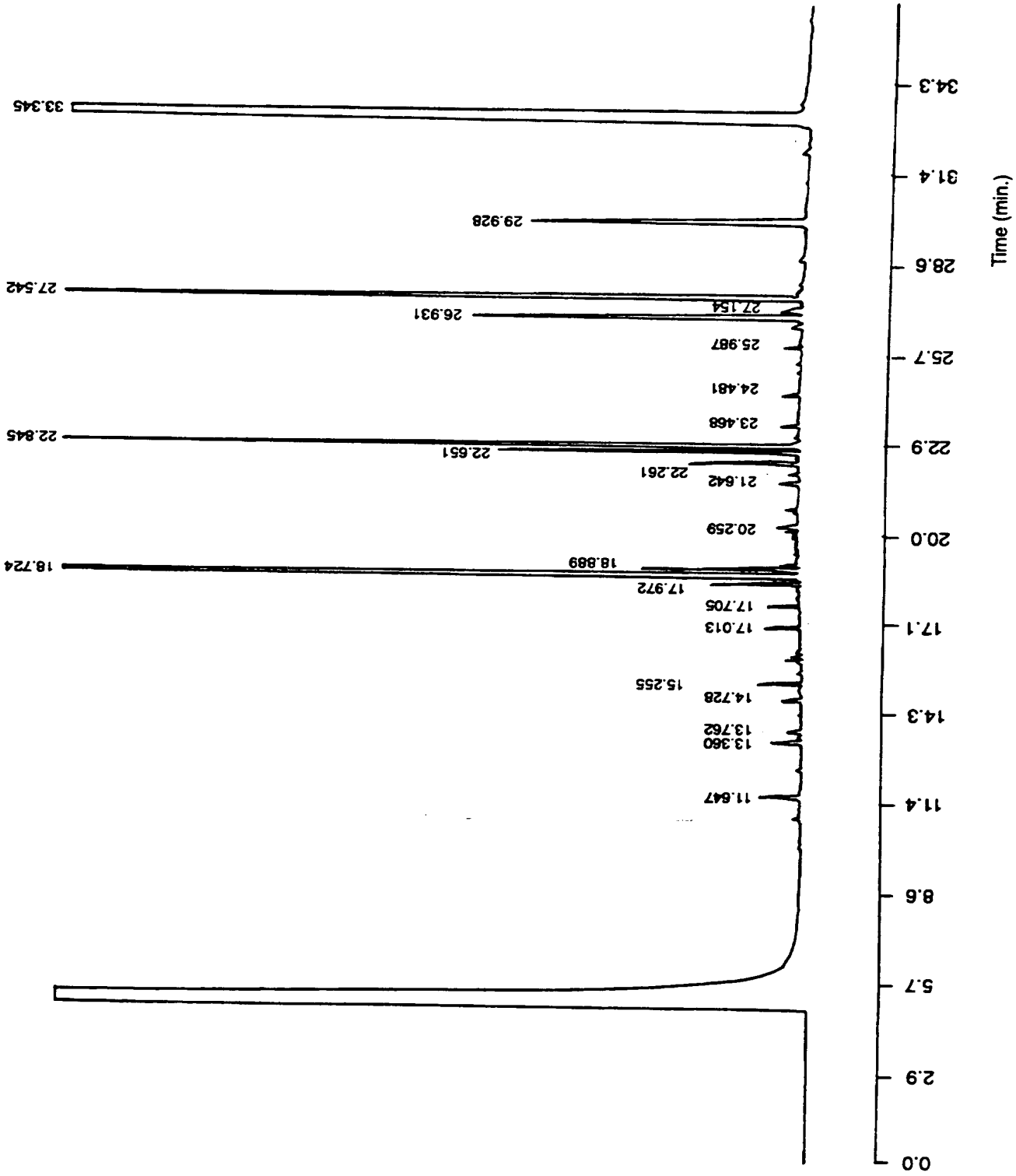


FIGURE 5

GC profile of RPFA-4 (RP-HPLC time window 30.8-40.4 mins)

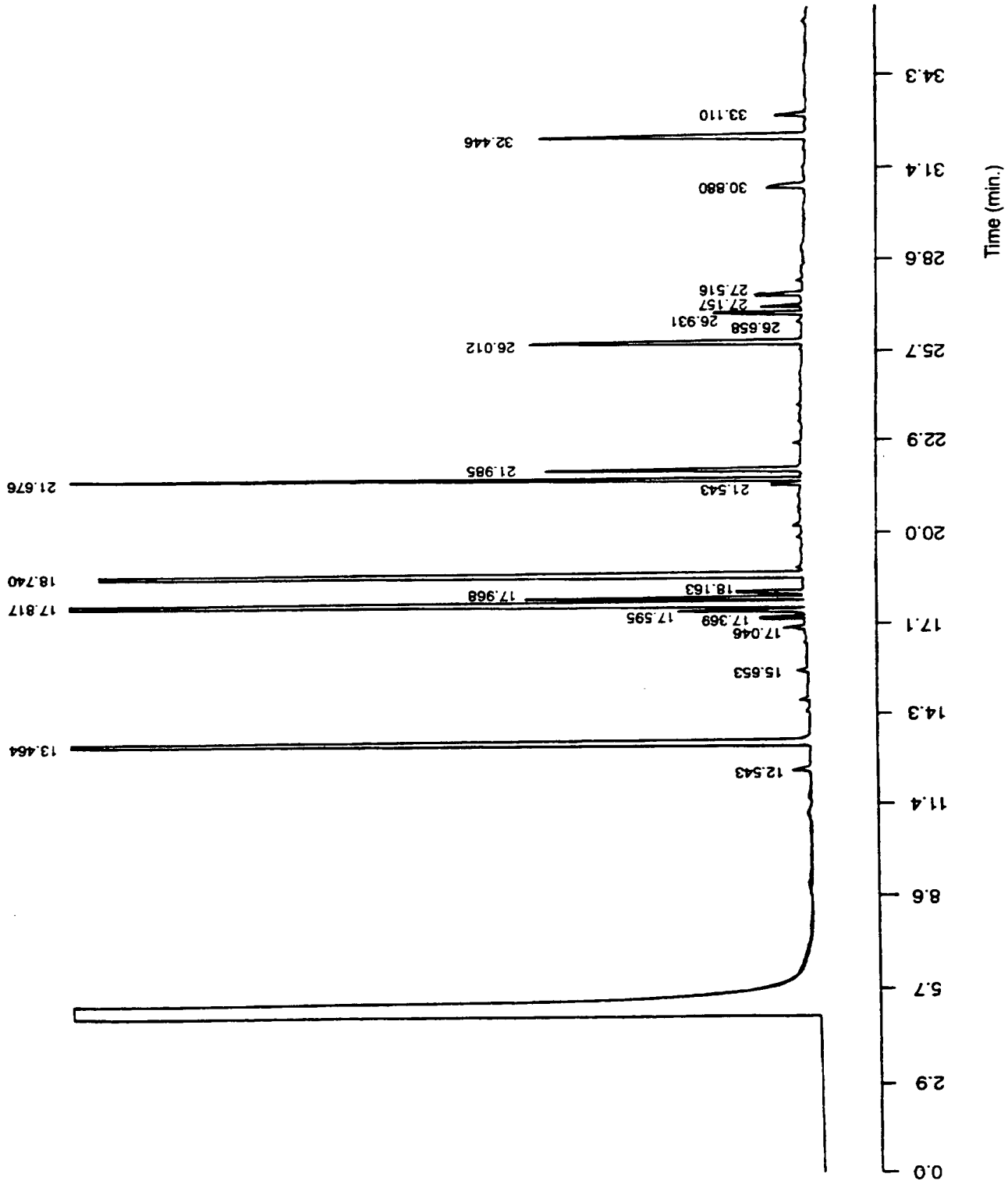


FIGURE 6

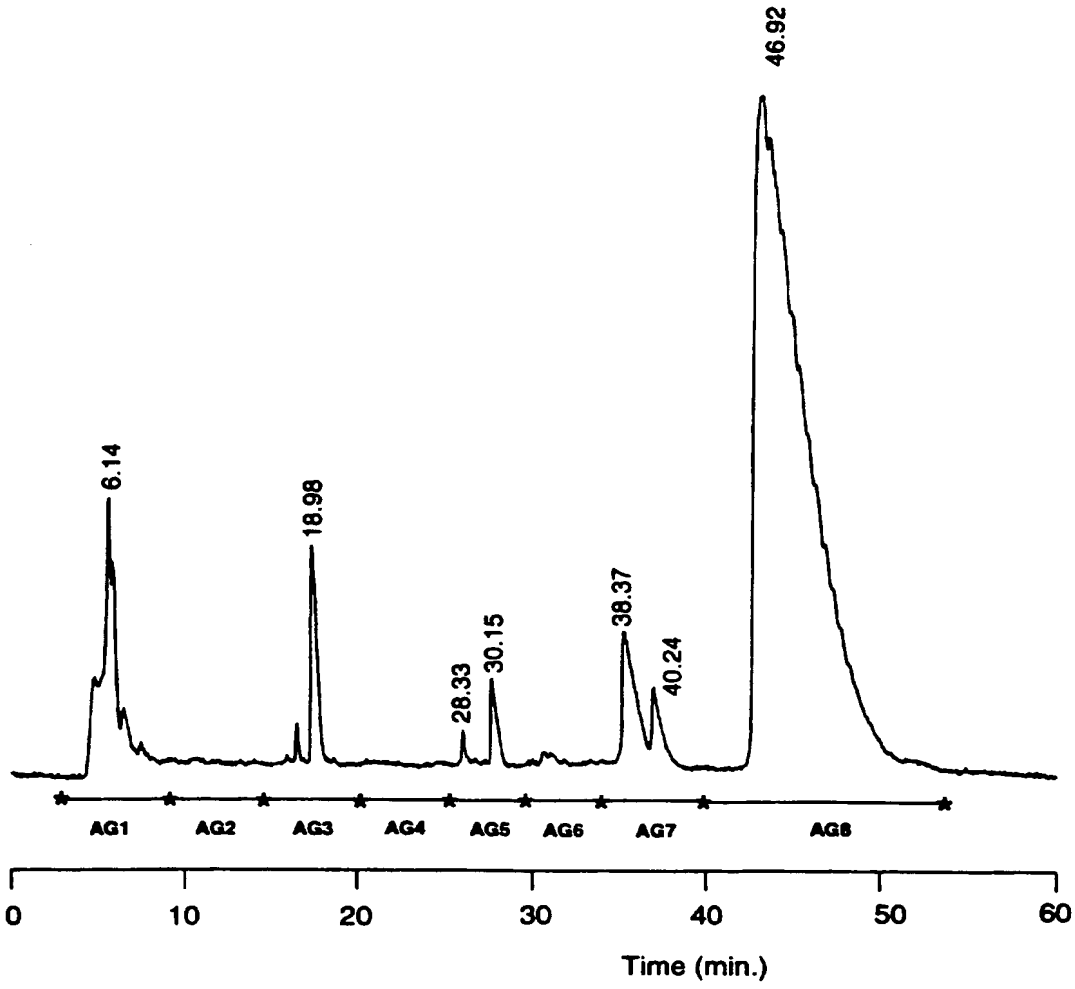

46.92
6.14
18.98
28.33
30.15
38.37
40.24
AG1
AG2
AG3
AG4
AG5
AG6
AG7
AG8
0
10
20
30
40
50
60
Time (min.)

FIGURE 7

GC profile of AG5 (Ag-HPLC time window 24.0-26.0 mins)

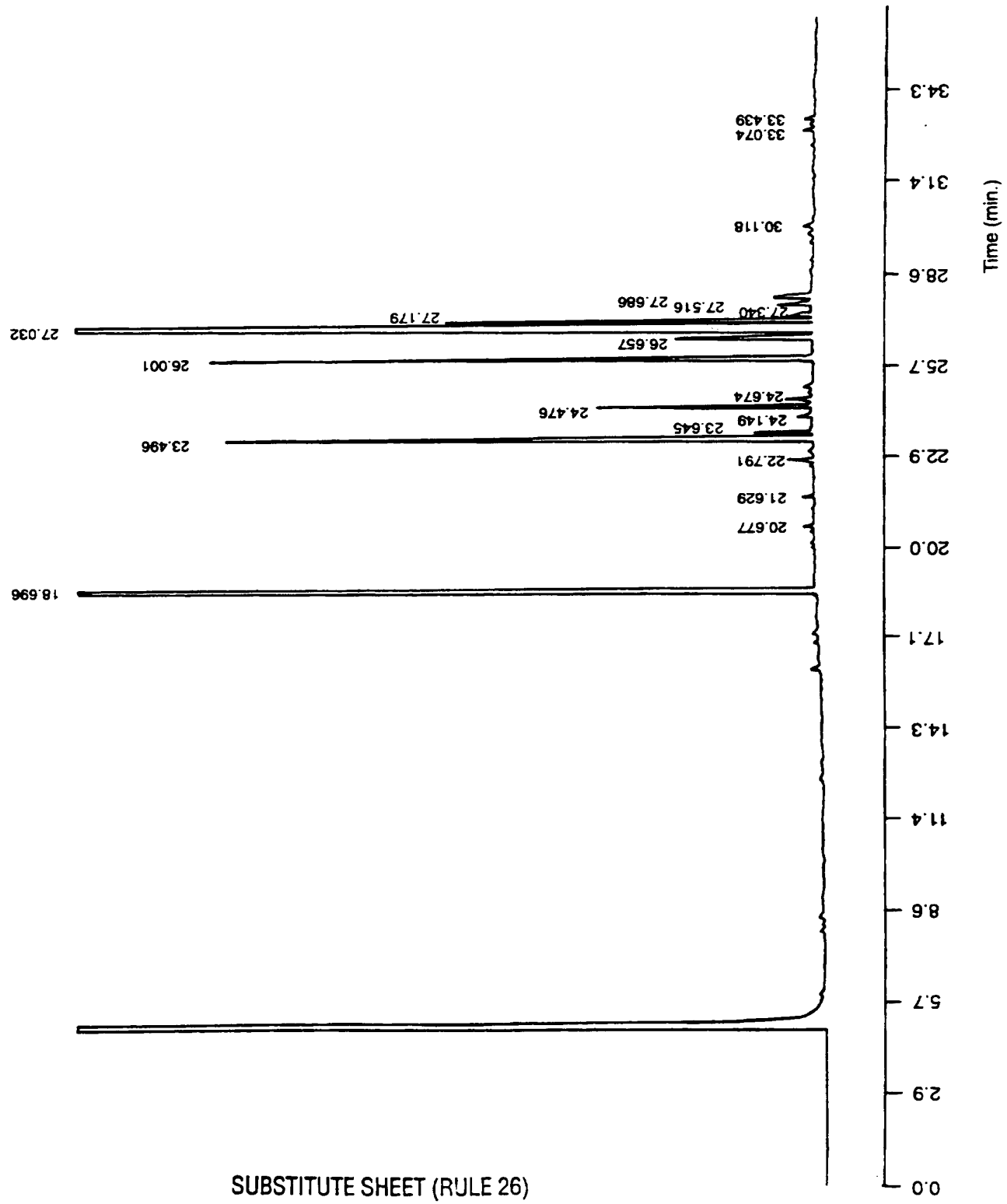


FIGURE 8

GC profile of AG6 (Ag-HPLC time window 26.0-33.7 mins)

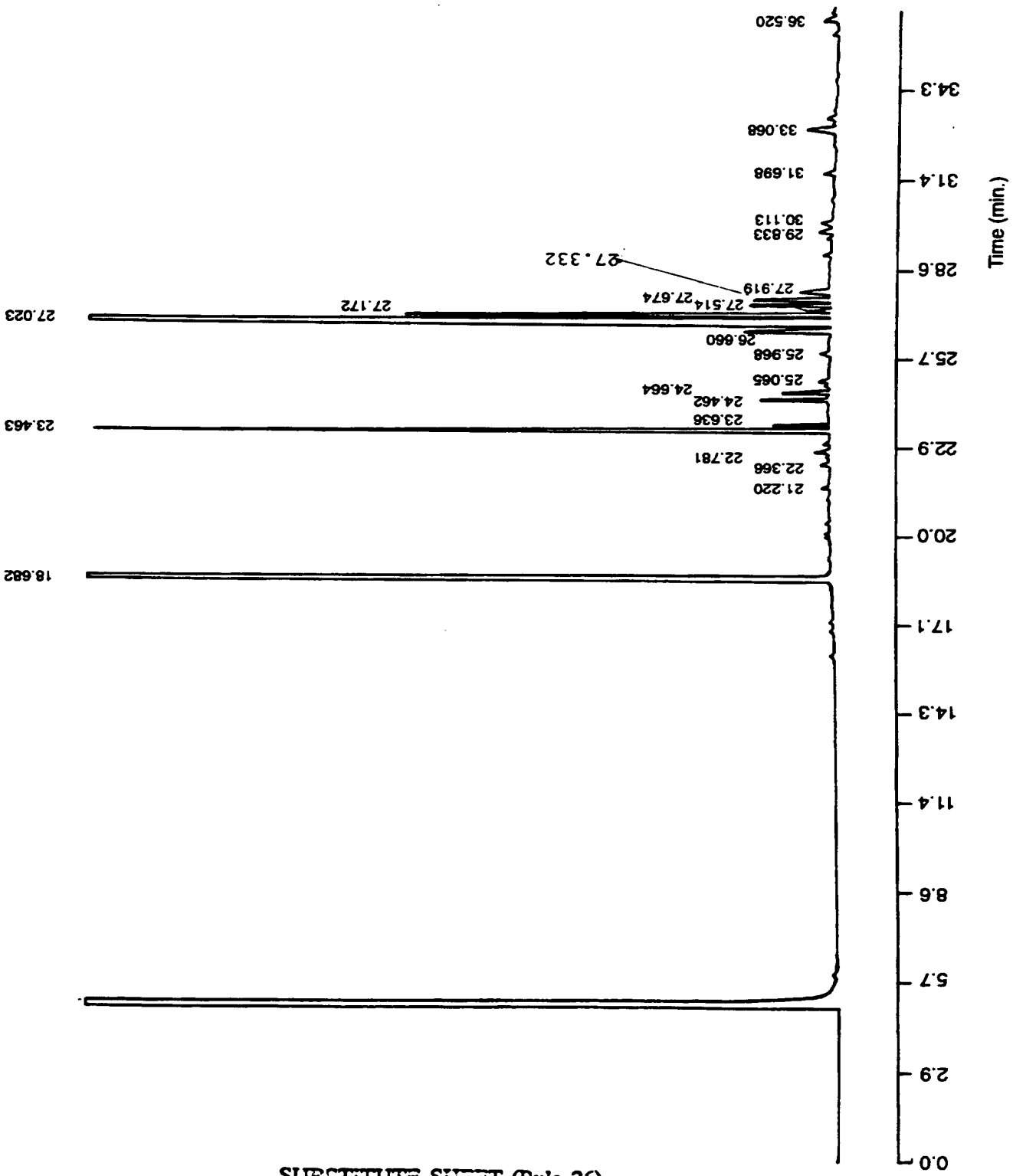


FIGURE 9
Molecular ion spectrum of fraction AG5 corresponding to peak at 23.469 mins identified as C18 .4

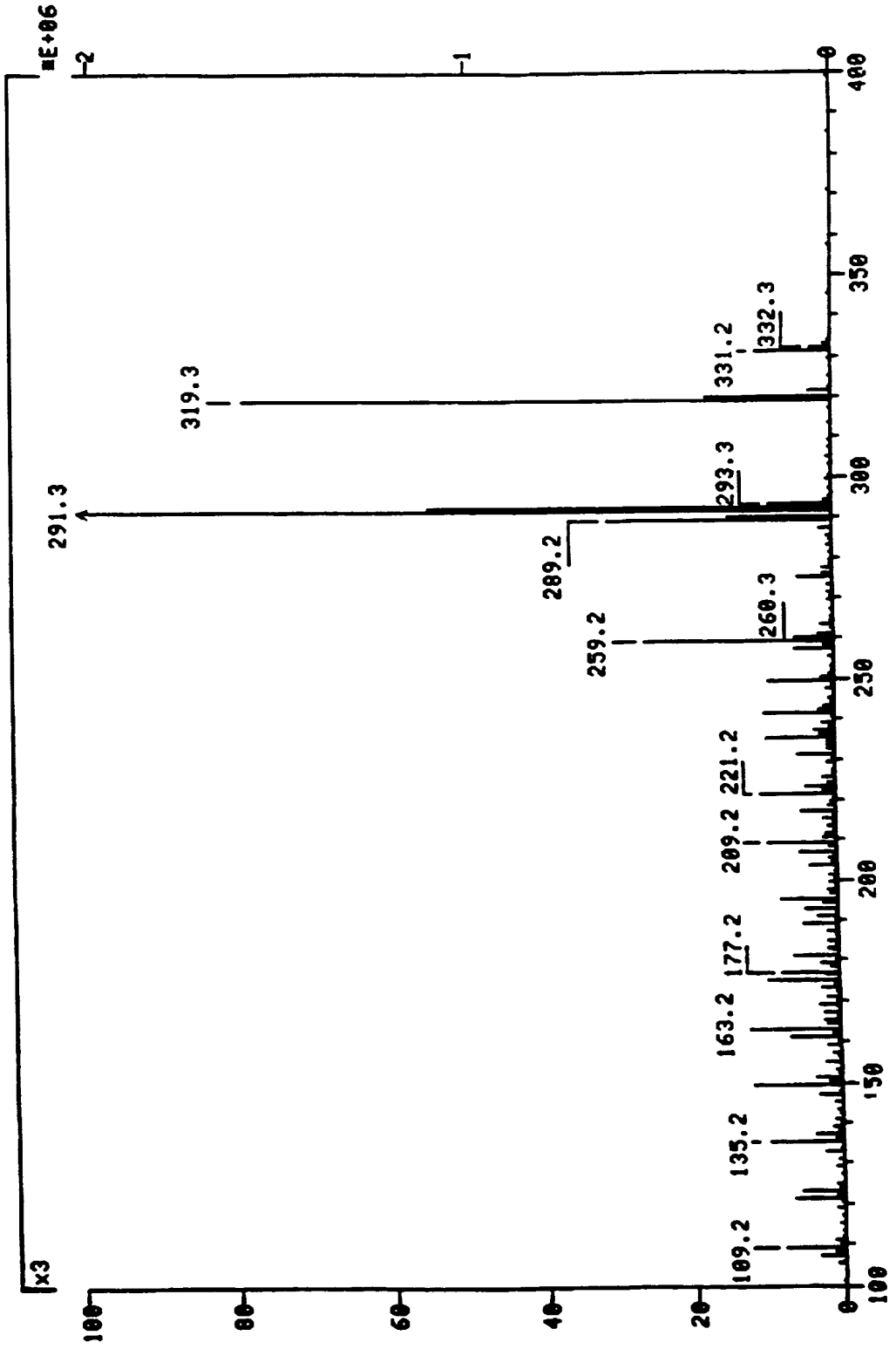


FIGURE 10

Molecular ion spectrum of fraction AG5 corresponding to peak at 24.476 mins, identified as C19:4.

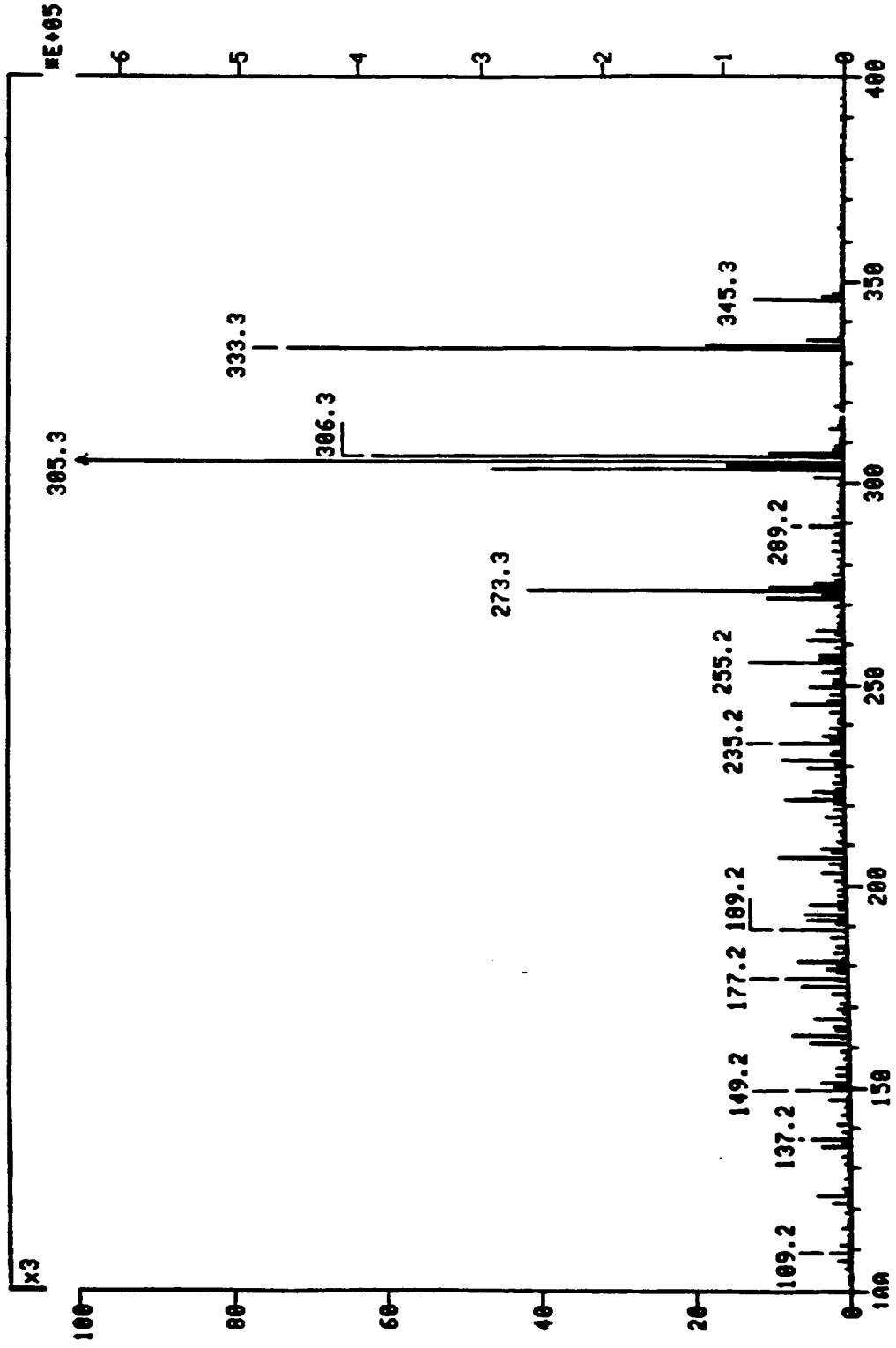


FIGURE 11

Molecular ion spectrum of fraction AG5 corresponding to peak at 26.001 mins, identified as C20:4 (Arachidonic acid methyl ester).

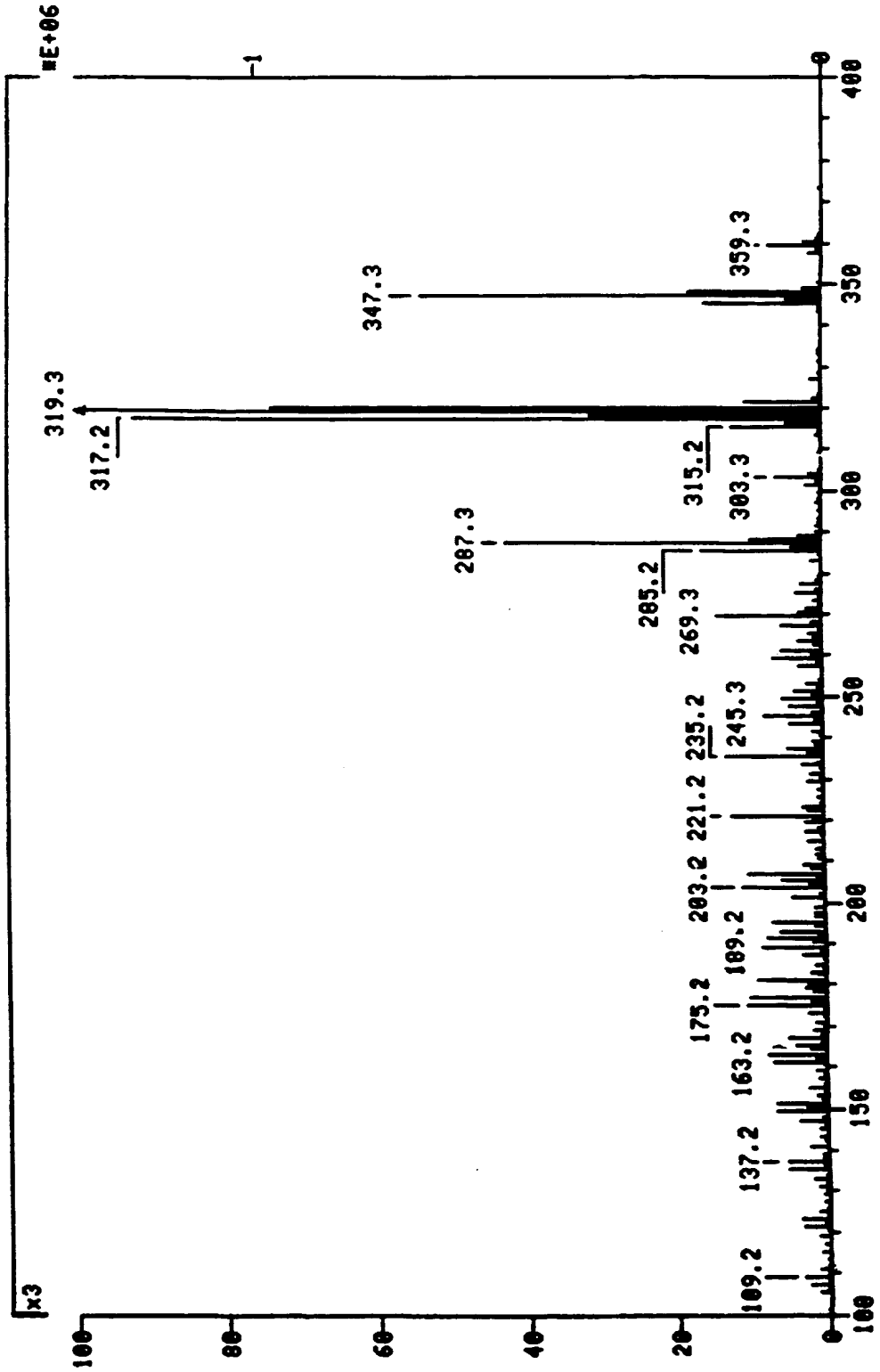


FIGURE 12

Molecular ion spectrum of fraction AG5 corresponding to peak at 27.032 mins, identified as C20:4.

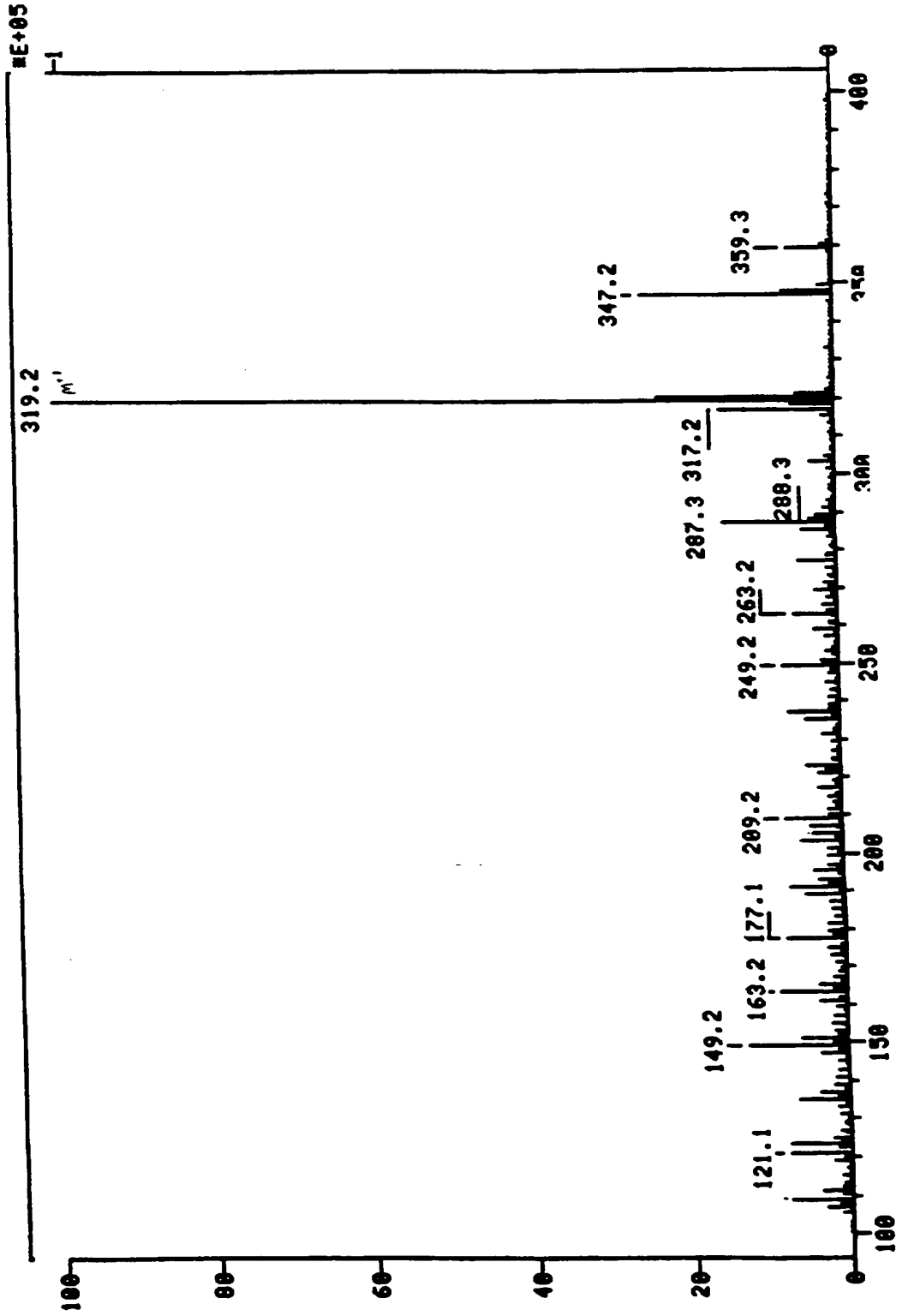


FIGURE 13

Molecular ion spectrum of fraction RPFA-3 corresponding to peak at 27.542 mins, identified as C20:5 (Eicosapentaenoic acid methyl ester).

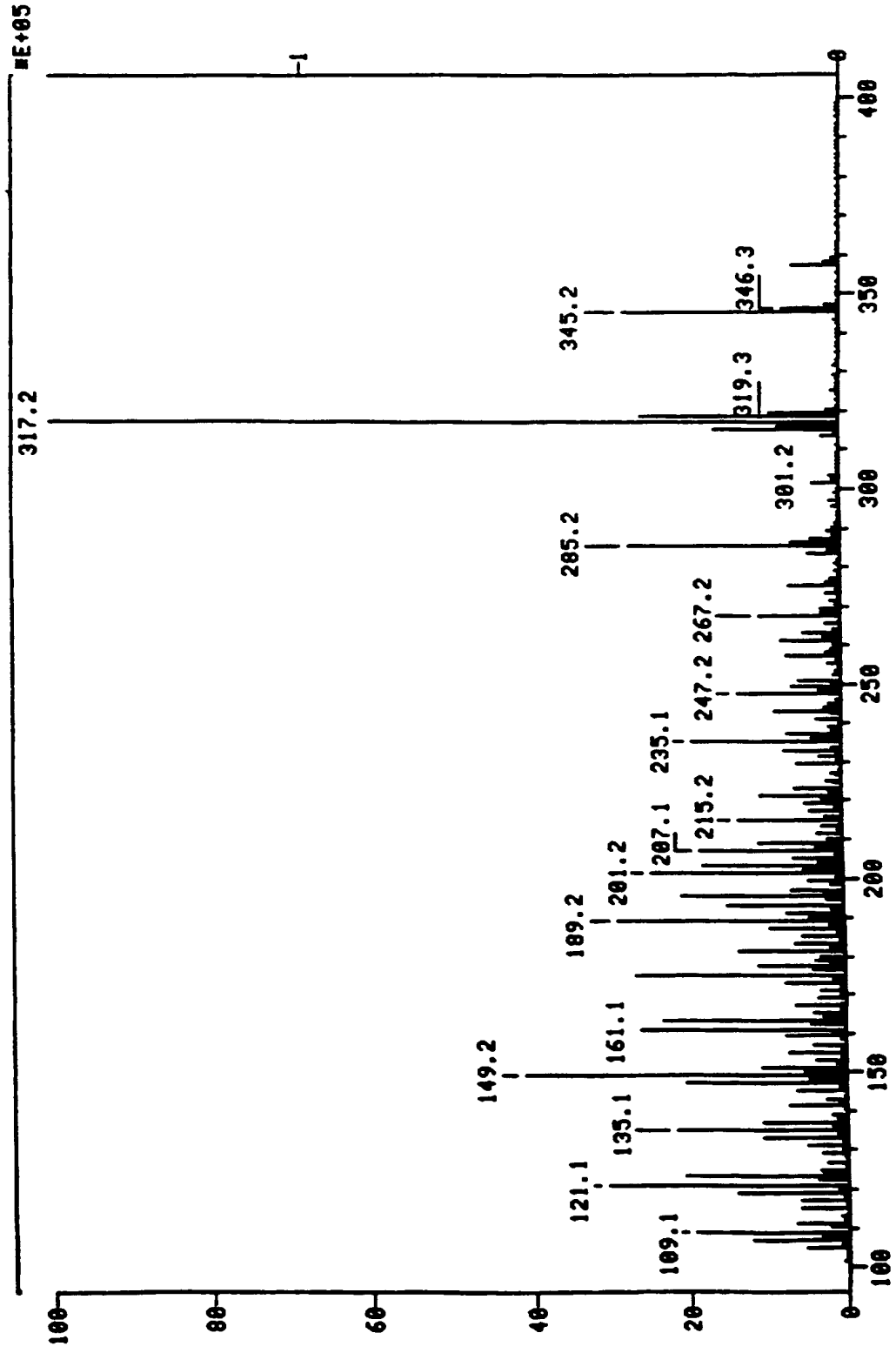
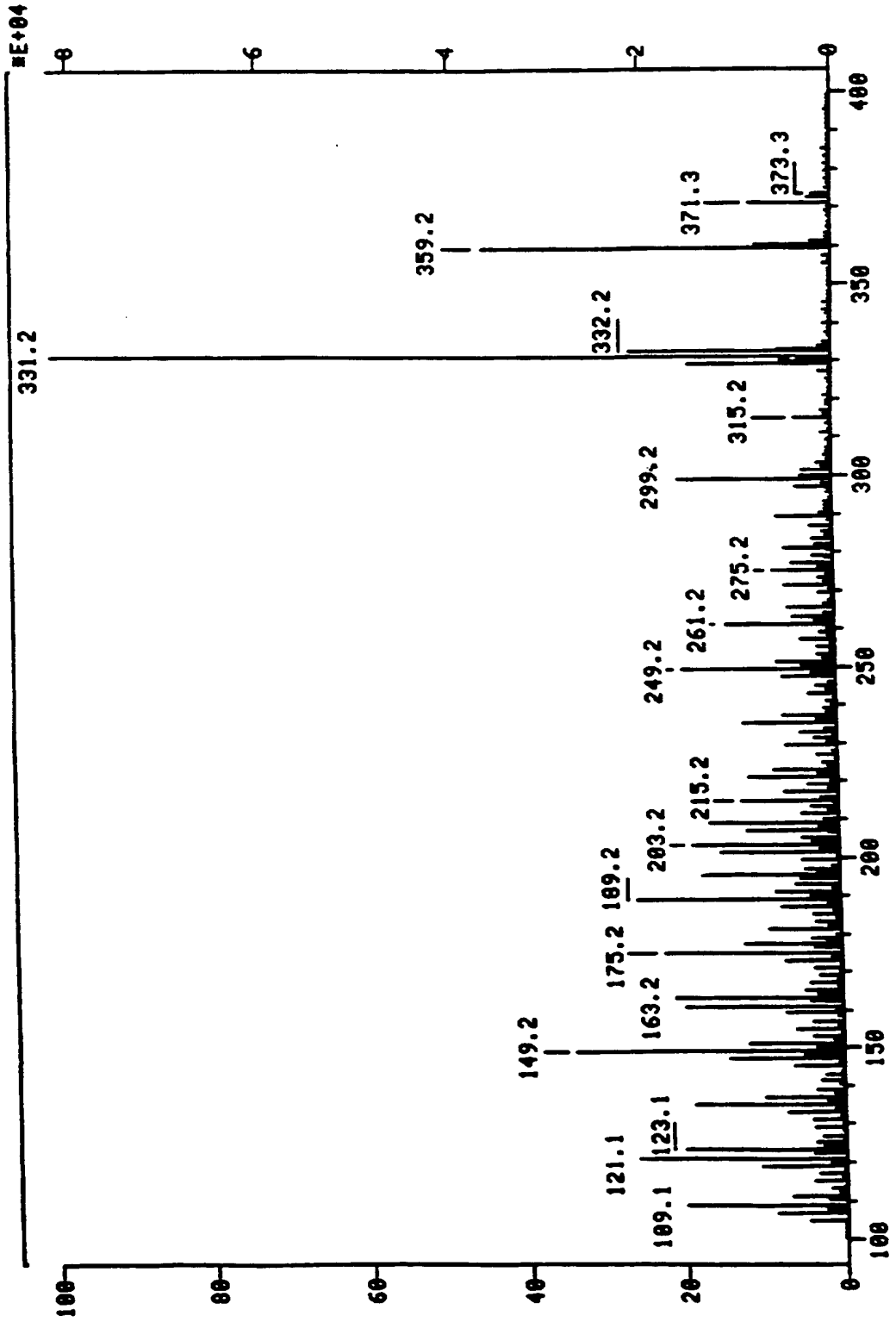


FIGURE 14

Molecular ion spectrum of fraction RPFA-3 corresponding to peak at 29.928 mins, identified as C21:5.



INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 95/00485

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C07C 57/03, 69/587, 233/09; A61K 31/20, 35/56, C11C 1/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC C07C 57/03, 57/02

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU: IPC as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DERWENT: PERNA OR MYTILUS
JAPIO: PERNA OR MYTILUS
CHEMICAL ABSTRACTS: (PERNA OR MYTILUS) AND (OIL: OR FAT: OR LIPID:); STN STRUCTURE SEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,Y	Arzneimittel Forschung/Drug Research, Volume 30 No. (ii), 1980, Editio Cantor, Aulendorf, Germany, K D Ramsford et al "Gastroprotective and anti-inflammatory Properties of Green Lipped Mussel (Perna Canaliculus) Preparation" pages 2128-2132 - see especially pages 2129 and 2131	1, 6, 8, 9, 12
Y	EP,A, 10061 (STUART JOHN McFARLANE & JOHN ERIC CROFT) 16 april 1980 see especially page 2	1, 8, 9, 12
Y	WO,A, 85/05033 (JAMES MEREDYTH BROADBENT & YOSHIKI KOSUGE) 21 November 1985 see especially pages 5 and 6	1, 8, 9, 12

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

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"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search
24 October 1995

Date of mailing of the international search report
6 NOVEMBER 1995

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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 95/00485

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Lipids, Volume 27 No. 7, 1992, American Oil Chemists Society, Champaign, Illinois, USA Ikuo Ikeda et al, "5c, 11c, 14c - Eicosatrienoic Acid and 5c, 11c, 14c, 17c - Eicosatetraenoic Acid of Biota Orientalis Seed Oil Affect Lipid Metabolism in the Rat" pages 500-504 whole document	1, 8, 12, 13-15, 16
X	Yukagaku/Journal of the Japan Oil Chemists Society, Volume 35 No. 7, 1986, Tokyo, Japan, toru Takagi et al, "Fatty Acid composition of Bivalves from Japanese Waters" pages 517-521 see especially page 520	1, 13-15
X	Yukagaku/Journal of the Japan Oil Chemists Society, Volume 31 No. 9, 1982, Tokyo, Japan, Yutaka Itabashi et al "Cis-5-Olefinic Nonmethylene-Interrupted Fatty Acids in Lipids of Seeds, Arils and Leaves of Japanese Yew", pages 574-579 see especially page 576	1, 13-15
X	Lipids, Volume 21, No. 9, 1986, American Oil Chemists Society, Champaign, Illinois, USA, Toru Takagi et al, "Fatty Acids in Echinoidea: Unusual cis-5-Olefinic Acids as Distinctive Lipid Components in Sea Urchins", pages 558-565 see whole document	1, 13-15
X	Biochemical Journal, Volume 291, No. 3, 1993 Biochemical Society, London, england, Javier Naval et al., "Alternative route for the biosynthesis of polyunsaturated fatty acids in K562 cells", pages 841-845 see especially pages 842 and 844	1, 13-15
X	Biochimica et Biophysica Acta, Volume 1167, No. 2, 1993, Elsevier, Netherlands, Saeree Jareonkitmongkol et al., "Occurrence of two nonmethylene-interrupted 5 polyunsaturated fatty acids in a 6-desaturase-defective instant of the fungus Mortierella alpina 1S-4" pages 137-141 see especially page 138	1, 13-15
X	Comparative Biochemistry and Physiology, Volume 107B, No. 2, 1994, Permagon Press, London, Valery M Dembitsky et al., "Comparative Study of the endemic freshwater fauna of Lake Baikal-III. Phospholipid and fatty acid compositions of the amphiphod crustacean of the genus Enlimnogammarus", pages 317-323 see especially Tables 5 and 6	1, 13-15
X	JAOCS: Journal of the American Oil Chemists Society, Volume 65 No. 4, 1988, American Oil Chemists Society, Champaign, Illinois, USA M.S.F. Lie Ken Jie, et al., "Lipids in Chinese Medicine. Characterisation of all cis-5, 11, 14, 17-eicosatetraenoic acid in Biota orientalis seed oil and a study of Oxo-Furanoid Esters derived from Biota Oil", pages 597-600 see especially page 600	1, 14-16

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/ AU 95/00485

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JAOCS: Journal of the American Oil Chemists Society, Volume 61 No. 3, 1984, American Oil chemists Society, Champaign, Illinois, USA, J R Vickery et al., "The Fatty Acid Composition of Gymnospermae Seed Leaf Oils", pages 573-575 see especially page 573	1, 14, 15
X	Patent Abstracts of Japan, C-1159, page 63 JP,A, 5-276964 (SUNTORY LTD), 26 October 1993	1, 13, 14
A	Derwent Abstract Accession No. 89-333329/46, Class B05, DE,C, 3814047 (FRATZER) 16 November 1989	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.
PCT/AU 95/00485

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
EP,A,	10061	AU,B,	51107/79	US,A,	4455298	CA,A,	1134745
		WO,A,	80/00661	DE,C,	2965855	ZA,A,	79/5039
		JP,A,	55-147223				
WO,A,	85/05033	AU,B,	41764/85	GB,A,	2176701	AT,E,	506/91
		JP,A,	61-502463	NZ,A,	211928	DE,C,	3576277
		US,A,	4801453	EP,A,	179819		

END OF ANNEX